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14. ABSTRACT

CXCR4 is highly expressed in breast carcinoma cells and is essential for breast cancer metastasis to the lung. The molecular mechanisms of CXCR4-mediated breast cancer metastasis are poorly understood. In this project we aimed to test the hypothesis that Rac proteins are essential for CXCR4-mediated breast carcinoma cell proliferation and survival, thereby contributing to breast cancer metastasis. In Task 1, we have investigated the role of Rac proteins in CXCR4-regulated breast carcinoma cell proliferation and survival in vitro. We have focused our analysis on a novel splice form of Rac1 that is induced during breast cancer progression. We have shown that Rac1b is induced under stress-related conditions, including hypoxia, ionizing radiation and serum starvation and that Rac1 and Rac1b play distinct roles in cell proliferation and cellular signaling events. In Task 2, we planned to determine the contribution of Rac proteins to breast cancer metastasis in vivo, but we had to terminate this line of research due to technical difficulties.

15. SUBJECT TERMS
Cancer cell biology, metastasis models, chemokines, Rac1, Rac1b, Rac3, CXCL12, CXCR4, proliferaton, survival, RNA interference

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INTRODUCTION

This project examines the roles of the small GTPases Rac1 and Rac3 in CXCR4-mediated metastasis of breast carcinoma cells. CXCR4 is highly expressed in breast carcinoma cells and is the receptor for CXCL12, a chemokine that is produced in abundance in organs that are targeted by metastatic breast cancer, such as lung, liver and bone. Rac1b is a splice form of Rac1 that is constitutively active and expressed in breast cancer tissue and therefore the specific role of this splice variant in CXCR4-mediated breast carcinoma metastasis is examined separate from the role of Rac1 itself. The first aim was to determine the specific roles of Rac1, Rac1b and Rac3 in CXCL12-stimulated carcinoma cell survival and proliferation as well as in downstream signaling events. The second aim was to examine the roles of Rac1, Rac1b and Rac3 in two animal models of breast cancer metastasis, the first using orthotopic mammary fat pad-implanted human breast carcinoma cells (spontaneous metastasis) and the second tail vein-injected cells (experimental metastasis).

Notes:

1) Tasks 1 and 2, as outlined in the Body of the report below, refer to the Tasks described in the revised Statement of Work, effective June 8, 2006.
2) The time period of the grant has been extended to 12/31/08 under a No Cost Extension (Amendment F00002, 5/23/08).

BODY

Task 1. To determine the role of Rac proteins in CXCL12-regulated functions in breast carcinoma cells *in vitro*.

Task 1a: Determine the contribution of Rac1 and Rac3 to CXCL12-stimulated proliferation and survival of breast carcinoma cells.

Accomplishment of this task has been strongly hampered by the poor responsiveness of metastatic breast carcinoma cells that we have been able to test to CXCL12. Thus, in the first year of the grant, we examined a number of MBA-MD-231 cell lines (obtained from ATCC or several colleagues in the field) but none of these showed significant expression levels of CXCR4 (report_06-Fig. 1). Subsequently, we obtained mammary fat pad-isolated MDA-MB-231 (termed T231) from Dr. Nakshatri (U. Indiana). However, even though these cells expressed robust levels of CXCR4, they were still poorly responsive to CXCL12 with respect to proliferation, survival and invasion. During the second and third year of the project, we therefore extensively characterized the responsiveness of BT474 cells to CXCL12. These cells express relatively moderate levels of CXCR4 and to some extent, they do respond to CXCL12, but this response is very modest (report_07-Fig. 1A, B) and turned out to be quite variable and therefore difficult to reproduce. We therefore decided to terminate these efforts and focus on the functional characterization of Rac1b in breast carcinoma cells (Tasks 1c and 1d).

Task 1b: Determine whether Rac1 and Rac3 are activated by CXCL12 in breast carcinoma cell lines.

As for task 1a, performance of this task also has been hampered by the lack of CXCL12-responsive metastatic breast carcinoma cells. Although we found that CXCL12 indeed enhances Rac1 activation in T231 cells by application of CXCL12 (report_06-Fig. 3), this effect also was very modest.

Task 1c: Determine the contribution of Rac1b to CXCL12-stimulated proliferation and survival of breast carcinoma cells.

Rac1b is a constitutively active splice form of Rac1 that does not bind to RhoGDI, a protein that keeps Rac proteins in the cytosol in an inactive GDP-bound state [1;2]. Rac1b is identical to Rac1, except for a 19 amino acid insertion downstream of the Switch II region. A critical role for Rac1b in breast cancer progression is supported by the finding that Rac1b is induced during malignant transformation of mammary epithelial cells and is involved in the pathway that causes oxidative damage to DNA and genomic instability [3]. The main splice form of Rac1 is constitutively and ubiquitously expressed and therefore is termed Rac1.

We obtained 4 siRNA oligos that target independent regions of the 19 aa insert in Rac1b. Two of the 4 oligos (Rac1b-2 and Rac1b-3) are similar in their knockdown efficiency (report_07-Fig. 2) and consistently show similar effects in most assays that we performed

and we therefore have concentrated on these oligos. The Rac1 oligos that we designed target both Rac1 and Rac1b. In order to be able to study the function of the Rac1 protein without affecting expression of Rac1b, we also designed two Rac1-specific oligos that span the sequence 5' and 3' of the Rac1b insert sequence. However, although both oligos efficiently inhibited the expression of Rac1, they also significantly reduced expression of Rac1b, thereby strongly limiting their usefulness.

Functional characterization of Rac1b showed that this Rac1 splice form is important for the formation of lamellipodia and the activation of the ERK and JNK MAP kinase cascades in ZR75-1 breast carcinoma cells (report_07-Figs. 3 and 6). Surprisingly, in these cells, simultaneous siRNA-mediated depletion of both Rac1 and Rac1b stimulated ERK and JNK, which indicates that Rac1 and Rac1b play antagonistic roles in the regulation of these MAP kinase pathways. The signaling intermediates that mediate these specific functions of the two Rac1 splice forms remain to be identified.

Rac1b is a constitutively active form of the Rac1 protein and therefore is likely to be regulated at the level of transcription. The only condition known thus far to induce Rac1b expression is the epithelial-mesenchymal transition (EMT) in murine breast carcinoma cells, we set out to study the role of Rac1b in this process that is caused by matrix metalloproteinase MMP3 or MMP9 [3]. Thus, to study Rac1b under physiologically relevant conditions, we set out to identify human breast carcinoma cell lines that are appropriate for the functional characterization of Rac1b in conditions where Rac1b is induced during the EMT process. Interestingly, Rac1b is poorly expressed in MCF10A**,** a normal human breast epithelial cell line, and MCF10DCIS cells, a tumor-forming derivative of MCF10A cells, is undetectable in all the highly invasive breast carcinoma cell lines that we have tested (MDA-MB-231, MDA-MB-435 and BT549), but is highly expressed in two poorly invasive, well-differentiated cell lines (ZR75-1 and MCF7) (report_08-Fig. 1 and data not shown). Another well-differentiated breast carcinoma cell line T47D, displayed intermediate Rac1b expression level (data not shown).

We therefore first examined whether Rac1b is induced during EMT in T47D cells. We found that EMT-like changes (cell-cell dissociation and cell elongation) induced by either addition of MMP9 or MDA-MB-231-conditioned medium significantly upregulated Rac1b (report_08-Fig. 2). However, contrary to what has been claimed in the literature [3], we could not find any evidence that Rac1b is necessary for the early stage of MMP-induced EMT (report_08-Fig. 3). Thus, Rac1b may play a role at a later stage of EMT, or subsequent events, such as migration and invasion.

Interestingly, Rac1b is also induced by serum starvation (report_08-Fig. 2), indicating that Rac1b can be induced by stress conditions. Thus, reasoning that Rac1b may not be important for the EMT process itself, but could protect the cell against the stress that is caused by the EMT process, we extended these observations to other stress conditions that are clinically relevant, hypoxia and ionizing radiation. We found that both hypoxia and application of $CoCl_2$, which mimics hypoxic conditions, significantly induced Rac1b expression in MCF10ADCIS cells (Figure 1A, B). Ionizing radiation (IR) also strongly induced Rac1b expression (Figure 2).

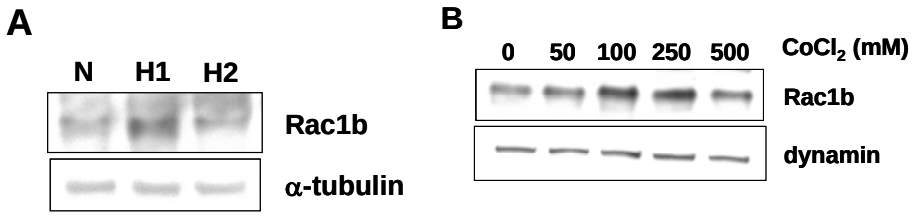

Figure 1: Rac1b is induced by hypoxia (A) and $CoCl_2$ (B).
A. MCF10DCIS cells were exposed to normoxia (N, 21% O_2) or hypoxia for 24h (H1, 1% O_2) or 48h (H2, 1% O_2). Cell lysates were prepared, proteins (20 μg cell lysate) separated on a 12% gel (Pierce) and transferred to PVDF membrane (Bio-Rad). The blots were probed with antibodies to Rac1b (1:1000) and α-tubulin (1:5000) and the bands visualized using SuperSignal Pico (Pierce). **B**. MCF10DCIS cells were treated with increasing concentrations of cobalt chloride (Sigma, 255599) for 24 h. Rac1b was visualized as in Fig. 1A, except that dynamin II (Santa Cruz, C-18; 1:1000) was used as loading control. The results are representative of respectively two (A) and three (B) independent experiments.

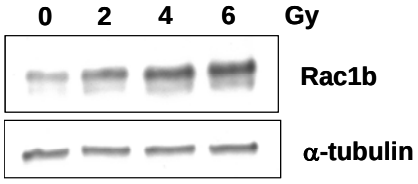

Figure 2: Rac1b is induced by ionizing radiation.
MCF10DCIS cells were exposed to increasing doses of IR for 48h. Cell lysates were prepared were prepared and analyzed as in Fig. 1. The results are representative of two independent experiments.

We next proceeded to test whether knockdown of Rac1b would interfere with IR or hypoxia-induced responses. However, somewhat surprisingly, we found that the siRNA transfection process strongly inhibited the upregulation of Rac1b, both by IR and hypoxia (data not shown). Several attempts to optimize the transfection process (using different transfection reagents, lowering the siRNA concentration), did not improve this condition. Thus, although we have convincingly shown that Rac1b expression is upregulated by a number of different stress-inducing conditions, thus far, we have been not able to identify a role for Rac1b in any of these stress responses.

EMT is thought to occur at early stages of breast cancer progression, i.e. DCIS-Stage I [4] and Rac1b predominantly appears to be regulated at the level of expression. Moreover, the restricted expression of Rac1b in well-differentiated cells may make Rac1b an interesting marker for a subset of breast cancers. We therefore set out to determine Rac1b expression levels in breast tumor tissue corresponding to various stages

of tumor progression. To this end, we have generated affinity-purified anti-peptide polyclonal antibodies (raised against the Rac1b insert region) in collaboration with Millipore. Unfortunately however, our immunohistochemical studies of paraffin-embedded formalin-fixed breast carcinoma cells revealed significant staining in breast carcinoma cells that do not appreciably express Rac1b, preventing the use of the antibody in immunohistochemistry. This non-specificity is presumably due to the fact that the antibody also recognizes a higher molecular weight protein as revealed by western blotting.

Task 2. To determine the contribution of Rac1, Rac1b and Rac3 to CXCL12/CXCR4-mediated breast cancer metastasis.

Task 2a: Construction and production of viruses for retroviral transfection of shRNA.

In order to examine the role of Rac proteins in CXCL12/CXCR4-mediated breast cancer metastasis we have made a several unsuccessful attempts to establish stable breast carcinoma cell lines with decreased levels of expression of Rac1, using a number of different shRNA constructs. The likely reason for this failure is that Rac1-depleted cells proliferate significantly slower than control cells. As mentioned in the 2008 report, we considered to engineer cells that express Rac1-directed shRNA from an inducible promoter, but the inducible systems in human cells that we know of tend to be unstable. We therefore have decided to conserve resources and to focus on the functional analysis of Rac1b.

KEY RESEARCH ACCOMPLISHMENTS

We found that Rac1b is preferentially expressed in well-differentiated breast carcinoma cell lines and is induced under stress-related conditions, including hypoxia, ionizing radiation and serum starvation. Our results indicate that Rac1b is not necessary for early stages of epithelial-mesenchymal transition in breast carcinoma cells. We also found that Rac1 and Rac1b largely control distinct functions in breast carcinoma cells. Whereas Rac1 stimulates proliferation and the formation of intercellular junctions, Rac1b does not. Moreover, Rac1b stimulates ERK and JNK activation, whereas Rac1 inhibits the activation of ERK, JNK and AKT in the breast carcinoma cells that we have examined.

REPORTABLE OUTCOMES

None

CONCLUSION

The poor responsiveness of the breast carcinoma cell lines that we were able to examine to CXCL12 in vitro have made it exceedingly difficult to examine the role of Rac proteins in CXCL12-stimulated functions in breast carcinoma, which constituted a large part of Task 1. Technical difficulties with establishing stable Rac1-depleted cell lines also have prevented us from accomplishing Task 2. However, we have made significant progress toward the functional characterization of Rac1b in breast carcinoma cells. We found that Rac1 and Rac1b largely control distinct functions in breast carcinoma cells. Whereas Rac1 stimulates proliferation and the formation of intercellular junctions, Rac1b does not. On the other hand, Rac1b stimulates ERK and JNK activation, whereas Rac1 inhibits the activation of ERK, JNK and AKT in the breast carcinoma cells that we have examined. In addition, we found that Rac1b is preferentially expressed in well-differentiated breast carcinoma cell lines and is induced under physiological and therapeutic stress conditions, including hypoxia, ionizing radiation and serum starvation.

Although these results shed important new light on the distinct roles of the two Rac1 splice forms, further mechanistic information is needed to produce a manuscript for publication.

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APPENDICES
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